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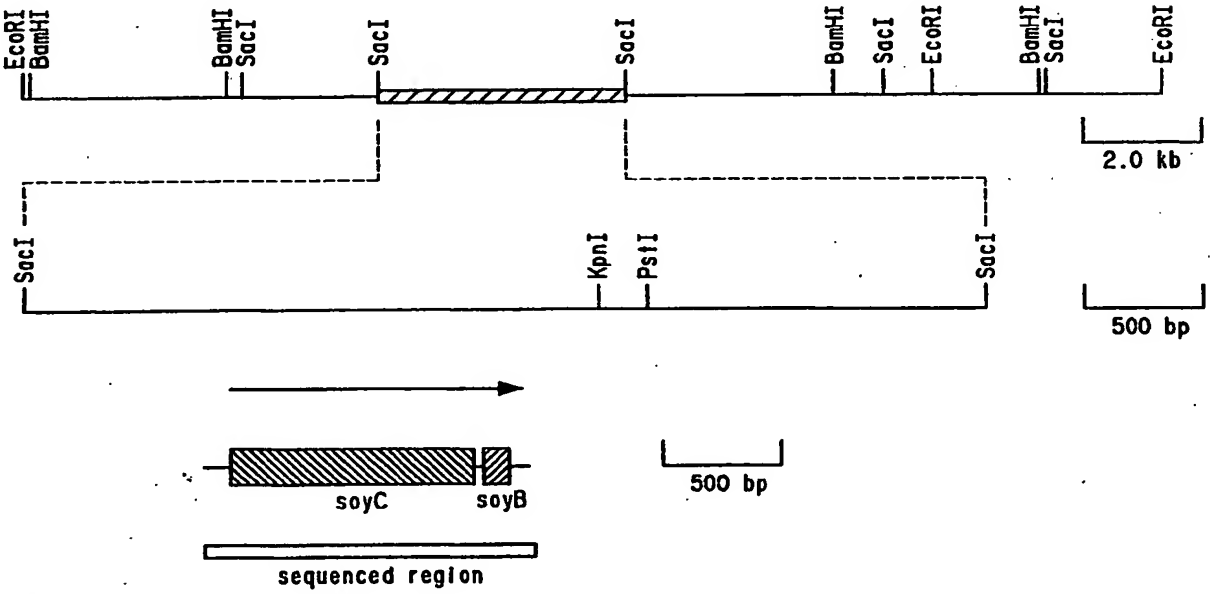

(57) Abstract

The present invention provides a method of making a recombinant organism capable of oxidizing organic chemicals by constitutive production of proteins capable of performing oxidation. A recombinant organism and a method of oxidizing organic chemicals are also provided. The present invention is useful in bioremediation to remove waste chemicals from the environment.

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TITLE

CONSTITUTIVE EXPRESSION OF P450SOY AND FERREDOXIN-SOY IN *STREPTOMYCES*, AND BIOTRANSFORMATION OF CHEMICALS BY RECOMBINANT ORGANISMS

FIELD OF THE INVENTION

This invention relates to recombinant bacteria of the genus *Streptomyces* capable of constitutive expression of cytochrome P450soy and the iron-sulfur protein that donates electrons to the cytochrome P450soy. These recombinant bacteria are useful in carrying out a number of important chemical conversions including biotransformation of HMPA and similar compounds.

BACKGROUND OF THE INVENTION

Cytochrome P450 (P450) is a term used for a widely distributed group of unique heme proteins which form carbon monoxide complexes with a major absorption band at wavelengths around 450 nm. These proteins are enzymes which carry out oxidase functions in a wide variety of mixed function oxidase systems involved in biosynthesis and catabolism of specific cell or body components, and in the metabolism of foreign substances entering organisms. Oxygenating enzymes such as P450 appear to be fundamental cellular constituents in most forms of aerobic organisms. The activation of molecular oxygen and incorporation of one of its atoms into organic compounds by these enzymes are reactions of vital importance not only for biosynthesis, but also for metabolic activation or inactivation of foreign agents such as drugs, food preservatives and additives, insecticides, carcinogens and environmental pollutants.

In eukaryotic systems P450, and P450 dependant enzymes are known to act on such xenobiotics and pharmaceuticals as phenobarbitol, antipyrine, haloperidol and prednisone. Known substrates of

environmental importance include compounds such as DDT, and a variety of polychlorinated biphenyls and polyaromatic hydrocarbons, as well as other halogenated compounds, including halobenzenes and chloroform.

Hexamethylphosphoramide (HMPA) is a compound that was used heavily by industry in the mid-1970's in the production of aramid fibers and as a general solvent. HMPA is a known carcinogen and has been found to be one of the contaminants at various industrial and chemical waste sites. Studies focusing on the mammalian biodegradation of HMPA are few but it has been found that microsomal P450 isolated from rat liver and nasal mucosa will demethylate HMPA. Longo et al., Toxicol. Lett. 44:289 (1988).

In microbial systems cytochrome P450 is known to oxidize many of the same xenobiotic substrates as in eukaryotic systems and thus can be targeted as possible indicators for the presence of toxic compounds in the environment. One of the earliest reports of xenobiotic transformation was by the bacterium *Streptomyces giseus* which is known to contain the gene for the expression of cytochrome P450. This transformation involved the conversion of mannosidostreptomycin to streptomycin. Sariaslani et al., Developments in Industrial Microbiology 30:161 (1989). Since then these reactions have been observed with compounds ranging from simple molecules such as benzene to complex alkaloids (such as vindoline and dihydrovindolin, codein, steroids, and xenobiotics such as phenylhydrazine, ajmaline and colchine. Sariaslani et al., Developments in Industrial Microbiology 30:161 (1989).

Genetically engineered microorganisms with the ability to express the P450 gene offer several potential advantages. Such microorganisms might be designed to express precisely engineered enzymatic pathways that can

more efficiently or rapidly degrade specific chemicals. Development efforts are aimed largely at chemicals that are toxic or recalcitrant to naturally occurring bacterial degradation.

It has been shown that bacteria of the genus *Streptomyces*, when properly induced, are capable of producing both cytochrome P450soy and the iron-sulfur protein (ferredoxin-soy) that donates electrons to cytochrome P450soy. Sariaslani et al., Biochem. Biophys. Res. comm. 141:405 (1986) The induction procedure involves growing the bacteria in a medium comprising an inducer such as soybean flour, genistein or genistin.

The method of Sariaslani et al. for producing P450 is useful however, the need to utilize an inducer such as soybean flour or a soybean flour-like substance to induce production of cytochrome P450soy in bacteria of the genus *Streptomyces* is a drawback. Such inducers are difficult to work with and represent an unknown variable in the field. Also, the need to induce the bacteria to produce the desired enzyme introduces an additional step in the method, making the method more complex.

There is a need for a simple method of bioremediating methylated phosphoric amides such as HMPA without the use of inducers to stimulate enzymatic activity. A simple method would be based on the use of bacteria capable of constitutive expression of cytochrome P450soy and the iron-sulfur protein that donates electrons to cytochrome P450. The cytochrome P450soy enzyme in *Streptomyces griseus* bears a resemblance in its oxidative reactions to the cytochrome P450 enzymes of mammalian liver microsomes and thus *Streptomyces griseus* could serve as an economical and convenient source of cytochrome P450 for indication of

the presence of hazardous chemicals as well as their possible bioremediation.

SUMMARY OF THE INVENTION

One aspect of the present invention provides recombinant bacteria of the genus *Streptomyces* capable of constitutive expression of cytochrome P450soy and the iron-sulfur protein that donates electrons to cytochrome P450soy.

Another aspect of the present invention provides a process for converting chemicals such as a mutagen or carcinogen into their oxidation products. The process comprises culturing recombinant bacteria of the genus *Streptomyces* capable of constitutive expression of cytochrome P450soy and the iron-sulfur protein in a culture medium containing the substance to be metabolized.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1a shows a consensus restriction map generated by *Bam*HI, *Eco*RI and SacI digestion of a 22kb region of the *Streptomyces griseus* encoding the P450soy gene. The flanking *Eco*RI restriction sites are from the vector polylinker.

Figure 1b shows the restriction map of the heme probe hybridizing 4.8kb *Sac*I fragment with the endonucleases unique to the M13mp18/19 vector polylinker.

Figure 1c shows the coding region for soyC and soyB.

Figure 2a shows the 1.7kb nucleotide sequence of *Streptomyces griseus* DNA containing both the soyC and soyB genes, and shows the 412 amino acid sequence for the P450-soy protein.

Figure 3 shows the insertion of the 4.8kb *Sac*I fragment containing soyC and soyB into pMM001. The

subsequent removal of the 4.8kb fragment from pMM001 and insertion into plasmid pCA0200 to generate pMM002.

Figure 4 is a Western blot of protein extracts of *Streptomyces griseus*, *Streptomyces lividans* C200 and *Streptomyces lividans* MM002 from the comparative example described below. It shows that the promotor on SoyB and SoyC is regulated in *Streptomyces lividans*.

Lane 1 = purified $P\text{-}450_{soy}$
Lane 2 = *Streptomyces griseus* extract grown on YEME medium
Lane 3 = *Streptomyces griseus* extract grown on 5x SBG medium
Lane 4 = *Streptomyces lividans* C200 extract grown on YEME medium
Lane 5 = *Streptomyces lividans* C200 extract grown on 5x SBG medium
Lane 6 = *Streptomyces lividans* MM002 (strain 35) extract grown on YEME medium
Lane 7 = *Streptomyces lividans* MM002 (strain 35) extract grown on 5x SBG medium
Lane 8 = *Streptomyces lividans* MM002 (strain 36) extract grown on YEME medium
Lane 9 = *Streptomyces lividans* MM002 (strain 36) extract grown on 5x SBG medium

Figure 5 shows the generation of pMM004. The insertion of the 4.8kb *Sac*I fragment containing soyC and soyB into pUC19 at the *Sac*I site to generate pMM005. A DNA fragment containing *Streptomyces griseus* soyC was amplified so that an *Eco*RI site was introduced at the 5' end. The new fragment was inserted into pUC19 to generate pMM003. A fragment from pCA0302 containing suaR was ligated to the fragment from pMM003 containing soyC, and a fragment from pMM005 containing soyB and pUC19.

Figure 6 describes the generation of pMM007 from pMM004 and pIJ702-322. Both pIJ702-322 and pBR322 are cut with SacI and ligated to a 4.1 kb SacI DNA fragment of pMM004 that contains suaP linked to soyC, B to generate pMM005. pMM005 is cut with Sph1 allowing the separation of the pBR322 and pMM007 plasmids from pMM006.

Figure 7 is a Western Blot of protein extracts from *Streptomyces griseus*, *Streptomyces lividans* C200 and *Streptomyces lividans* MM002 showing that *Streptomyces lividans* MM002 expresses P450soy constitutively.

Lane 1 = purified $P\text{-}450_{soy}$

Lane 2 = *Streptomyces griseus* extract grown on YEME medium

Lane 3 = *Streptomyces griseus* extract grown on 5x SBG medium

Lane 4 = *Streptomyces lividans* C200 extract grown on YEME medium

Lane 5 = *Streptomyces lividans* C200 extract grown on 5x SBG medium

Lane 6 = *Streptomyces lividans* MM007 extract grown on 5x SBG medium

Lane 7 = *Streptomyces lividans* MM007 extract grown on YEME medium

DETAILED DESCRIPTION OF THE INVENTION

In the context of this disclosure, a number of terms shall be utilized.

"Promoter" and "promoter region" refer to a sequence of DNA, usually upstream (5') to the protein coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at the correct site. Promoter sequences are necessary but not always sufficient to drive the expression of the gene.

A "fragment" constitutes a sequence of nucleic acid which can contain an entire gene, less than an entire gene or more than an entire gene.

"Regulation" and "regulate" refer to the modulation of gene expression controlled by DNA sequence elements located primarily, but not exclusively upstream of (5' to) the transcription start of a gene. Regulation may result in an all or non response to a stimulation, or it may result in variations in the level of gene expression.

The term "coding sequence" refers to that portion of a gene encoding a protein, polypeptide, or a portion thereof, and excluding the regulatory sequences which drive the initiation of transcription.

"Construction" or "construct" refers to a plasmid, virus, autonomously replicating sequence, phage or nucleotide sequence, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

"Transformation" is the acquisition of new genes in a cell after the incorporation of nucleic acid (usually double stranded DNA).

"Operably linked" refers to the chemical fusion of two fragments of DNA in a proper orientation and reading frame to be transcribed into functional RNA.

"Expression" as used herein is intended to mean the transcription and translation to gene product from a gene coding for the sequence of the gene product. In the expression, a DNA chain coding for the sequence of gene product is first transcribed to a complimentary RNA which is often a messenger RNA and, then, the thus

transcribed messenger RNA is translated into the above-mentioned gene product if the gene product is a protein.

"Translation initiation signal" refers to a unit of three nucleotides (codon) in a nucleic acid that specifies the initiation of protein synthesis.

"Plasmid" as used herein refers to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules.

"Restriction endonuclease" refers to an enzyme which binds and cuts within a specific nucleotide sequence within double-stranded DNA.

"ATCC" refers to the American Tissue Culture Collection depository located in Rockville, Maryland. The "ATCC No." is the accession number to cultures on deposit at the ATCC.

"NRRL" refers to the U.S. Department of Agriculture, Northern Regional Research Laboratories, located in Peoria, Illinois, and the "NRRL No." is the accession number to cultures on deposit at the NRRL.

The invention involves *Streptomyces* transformed with two genes from *Streptomyces griseus*: the soyC-encoding cytochrome P450soy and the soyB-encoding ferredoxin-soy that transfers electrons to P450soy. These two genes are transcribed by a constitutive promoter, suaP, from another *Streptomyces*, *Streptomyces griseolus*. These transformed *Streptomyces lividans* strains constitutively express metabolically active P450soy and thus can metbolize a variety of organic chemicals without having to be induced. The natural promoter for the soyC and the soyB genes, soyP, is not constitutive in *Streptomyces lividans*. This is different from two inducible cytochrome P450 systems (suaC and suaB, and subC and subB) from *Streptomyces griseolus* (ATCC 1176) that metabolize sulfonylureas.

The promoters for suaC and suaB, suaP, and the promotors for subC and subB, subP, while requiring induction in *Streptomyces griseolus*, are constitutively expressed when transformed into *Streptomyces lividans* (U.S. Patent Application 07/464,499 filed January 12, 1990).

The genes encoding cytochrome P450soy (soyC) and ferredoxin-soy (soyB) are contained on a part of a 4.8 kb SacI DNA fragment from *Streptomyces griseus* (ATCC 13273). Alternative sources of this DNA could be *Streptomyces griseus* (ATCC 10137) and *Streptomyces griseus* (ATCC 55185), which also contain proteins similar to, if not identical to cytochrome P450soy of Streptomyces griseus (ATCC 13273).

The DNA containing the soyC and soyB genes is operably linked to a promoter sequence, which is capable of constitutively transcribing soyC and soyB in strains of *Streptomyces* bacteria. The preferred source of this promoter is a 0.6 kb EcoRI-BamHI DNA fragment in pCAO302 from *Streptomyces griseolus* (ATCC 11796). This is the promoter for the suaC and suaB genes which code for cytochrome P450sua and ferredoxin-sua, respectively, in *Streptomyces griseolus* (ATCC 11796). Omer et al., J. Bacteriol. 172:3335(1990). Alternative sources for such a constitutive promoter include but are not limited to one of the promoters for the agarase gene of *Streptomyces coelicolor*, Buttner et al., Cell 52:599(1988), the promoters for the thiostrepton resistance gene from *Streptomyces azureus*, Janssen and Bibb Mol. Gen. Genet. 221:339 (1990), and the constitutive promoter for the *Streptomyces lividans* galactose operon, Fornwald et al., Proc.Natl. Acad. Sci. U.S.A. 84:2130 (1987).

The combination of a constitutive promoter operably linked to the soyC and soyB genes is then introduced into plasmid DNA capable of transforming *Streptomyces*.

The preferred plasmid is pIJ702. Katz et al., J. Gen. Micro. 129:2703(1983). Other plasmids that could be used include but are not limited to derivatives of pIJ101, Kieser et al., Mol. Gen. Genet. 185:223(1982)) and SCP2, Lydiate et al., Gene 35:223(1985). The plasmid is then cloned into a host *Streptomyces* strain. The preferred *Streptomyces* host is *Streptomyces lividans* JI1326. Other *Streptomyces* host strains that could be used include but are not limited to *Streptomyces griseus* (ATCC 10173), *Streptomyces griseus* (ATCC 13273), *Streptomyces coelicolor* A3(2) and *Streptomyces parvulus* (ATCC 12434).

Bacterial host strains used include *Streptomyces griseus*, ATCC 13273, *Streptomyces griseolus*, ATCC 11796, and *Streptomyces lividans*, JI1326 (ATCC 53939). The *Streptomyces* strains were cultured in the following four media: (1) liquid YEME (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1.0% glucose, 5mM $MgCl_2$); (2) 5xSBG (2% glycerol, 0.5% yeast extract, 2.5% soybean flour, 0.5% NaCl, 0.5% K_2HPO_4, pH7.0); (3) 1 x SBG (2% glycerol, 0.5% yeast extract, 0.5% soybean flour, 0.5% NaCl, 0.5% K_2HPO_4, pH7.0); and (4) trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD). Generally, the cultures should be maintained at temperatures between 20°-30°C, preferably between 25°-37°C, with the optimum growth temperature at about 28°-30°C. Cultures were grown by shaking at 28-30°C and cells were harvested by centrifugation at approximately 10,000 x g for 10-30 min. The pelleted cells were resuspended in DEP buffer (29.3 g/l $Na_2HPO4-12H_2O$ or 21.98 g/l $Na_2HPO4-7H_2O$, 2.62 g/l $NaH_2PO_4-H_2O$, 0.037 g/l Na_2 EDTA, 0.154 g/l Dithiothreitol) and sometimes repelleted.

The final pellets were resuspended in DEP buffer and broken in a French pressure cell at 20,000 psi. The

broken cells were centrifuged at approximately 40,000 x g for 30 minutes and the soluble protein fraction removed and its concentration determined using the BioRad protein assay (Biorad, Richmond, CA). Western blots were performed using the procedure described and the antibody to cytochrome P450soy. Trower et al., J. Bacteriol. 171:1781(1989).

The recombinant bacteria of the present invention are prepared using methods well known to those skilled in the art. For example, transformation of the DNA fragments containing the transcriptional promotor suaP, from the suaC and suaB genes of *Streptomyces griseolus*, upstream of the soyC and soyB genes of *Streptomyces griseus* into *Streptomyces lividans* is performed as described by Hopwood, D. A. et al., Genetic Manipulation of Streptomyces: A Laboratory Manual, The John Innes Foundation, Norwich, UK (1985). Cloning of these DNAs in *E. coli* is performed as described by Maniatis, T. et al., A Guide to Molecular Cloning, Cold Spring Harbor (1982). Restriction enzymes and DNA modification enzymes can be obtained from New England Bioloabs Inc. Beverly, MA. Taq DNA polymerase can be obtained from Cetus-Perkin Elmer Inc. Following the above procedures, recombinant bacteria *Streptomyces lividans* MM007 was generated from *Streptomyces lividans* JI1326.

The recombinant bacteria of the present invention may be employed to oxidize organic chemicals by culturing recombinant bacteria of the genus *Streptomyces* capable of constitutive expression of cytochrome P450soy and the iron-sulfur protein that donates electrons to cytochrome P450soy in a culture medium comprising the chemical to be oxidized. The product(s) of oxidation can be determined if required, by standard methods.

It is preferable to use a two stage culturing procedure. In stage one, the bacteria are grown in a

suitable culture medium for up to five days at a temperature between about 25° and 37°. In stage two, an aliquot of the stage one culture is transferred to fresh culture medium and maintained for up to five days. The most preferred culturing procedure is carried out by growing the bacteria in stage one at 28-30° for 3 days, transferring the bacteria to fresh medium and growing in stage two for one additional day at the same temperatures. The second stage culture is then used for the process of the present invention. That is, an aliquot of the substance to be oxidized is incubated with the 24 hr. old second stage cultures.

For example, a first stage culture is prepared by combining 0.5 ml of a spore preparation from *Streptomyces lividans* pMM007 with 25 ml of YEME medium, plus 50 ml of a 2.5M $MgCl_2$ solution and 62.5 µl of a 4 mg/ml stock solution of thiostrepton. This is then incubated at 28-29° for 72 hours in a gyrotary shaker. The second stage culture is prepared by adding a 2.5 ml portion of the first stage culture to 25 ml of fresh medium. Finally, 5 mg of the substance to be evaluated (e.g., benzo[a]pyrene or benzidine) is dissolved in a solvent such as dimethylsulfoxide (DMSO) and added to the 24 hr. old described second stage culture and incubated for an additional 1 to 10 days. Liquid substrates are added directly to the medium. Samples (5 ml) are periodically taken from these cultures and analyzed by standard methods for the presence of oxidation products.

Recombinant bacteria provided by this invention may be utilized to carry out many commercially important oxidation reactions, as will be recognized by those skilled in the art. The compounds which may be oxidized by the provided recombinant bacteria (and the oxidized compound resulting therefrom) include but are not

limited to the following: hexamethylphosphoramide (HMPA), pentamethylphosphoramide (PMPA), tetramethylphosphoramide (TetraMPA), trimethylphosphoramide, (TriMPA), 7-ethoxycoumarin (7-hydroxycoumarin); precocene II (precocene-diol); anisole (phenol, 2-OH anisole); benzene (phenol); biphenyl (4-OH biphenyl); chlorobenzene (2-OH chlorobenzene); coumarin (7-OH coumarin); naphthalene (1-OH naphthalene); trans-stilbene (4-OH stilbene, 4,4'-di-OH stilbene); toluene (2-OH toluene); glaucine (predicentrine, norglaucine); 10,11-dimethoxyaporphine (apocodeine, isoapocodeine); papaverine (6-desmethylpapaverine, 7-desmethylpapaverine, 4'-desmethylpapaverine); d-tetrandrine (N'-nortetrandrine); thalicarpine (hernandalinol); bruceantin (side chain alcohols, epoxide); vindoline (dihydrovindoline ether, dihydrovindoline ether dimer, dihydrovindoline ether enamine); dihydrovindoline (11-desmethyldihydrovindoline); leurosine (12'-hydroxyleurosine); and codeine (14-hydroxycodeine).

EXAMPLES

General Methods

Cloning and DNA sequencing of the soyC and soyB genes encoding cytochrome P450soy and ferredoxin-soy

Cytochrome P450soy was purified from *Streptomyces griseus* ATCC 13273 as described. Trower et al., J. Bacteriol. 171:1781(1989). Two similar forms of cytochrome P450soy were isolated. P450soyΔ, is derived from P450soy by *in vitro* proteolysis during isolation. Trower et al., J. Bacteriol. 171:1781(1989). Purified P450soy protein was alkylated with 4-vinylpyridine and 5 nanomoles of the alkylated cytochrome P450soy was digested with trypsin as described by Trower et al., J. Bacteriol. 171:1781(1989). The resulting peptide fragments were resolved by reverse phase high performance liquid chromatography as described by Trower

et al., J. Bacteriol. 171:1781(1989). One of the tryptic peptide fragments of cytochrome P450soy and one of the P450soyΔ protein were subjected to automated Edman degradation to determine the partial amino acid sequence of the protein/peptide. The NH_2-terminal sequence of the P450soyΔ protein is (Seq. No. 1):

Thr	Thr	Asp	Pro	Ala	Arg	Gln	Asn	Leu	Asp	Pro	Thr	Ser	Pro	Ala	Pro.
1				5					10					15	

The NH_2 terminal sequence of the tryptic peptide is (Seq. No. 2):

His	His	Leu	Ala	Phe	Gly	Phe	Gly	Val	His	Gln	Cys	Leu	Gly	Gln	Asn.
1				5					10					15	

A mixture of oligonucleotides that consist of possible DNA sequences that could encode the amino acids FGVHQCL (Sequence ID NO. 7) of the tryptic peptide was made. It consists of the following sequence: 5'- TTCGG(G or C)GT(G or C)CACCAGTGCCT- 3' (Sequence ID NO. 3-6). During synthesis of the oligonucleotide mixture, the two positions indicated as (G or C) consisted of an equal mixture of G or C and thus the oligonucleotide mixture consists of a total of four different species.

A DNA library was constructed in the vector EMBL4 using DNA from *Streptomyces griseus* ATCC 13272 using the procedures described. Omer et al., J. Bacteriol. 172:3335 (1990). The oligonucleotide mixture was [^{32}P]-end labeled using T4 polynucleotide kinase, Maniatis, T. et al., A Guide to Molecular Cloning, Cold Spring Harbor,(1982), and used to probe the EMBL4 library of *Streptomyces griseus* DNA as described in Maniatis, T. et al., A Guide to Molecular Cloning, Cold Spring Harbor,(1982), under the following conditions:

Prehybridization and hybridization were carried out in 6X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate) + 0.5% SDS at 50°C . Filters were washed twice in 6X SSC + 0.5% SDS at 50°C and once in 6X SSC + 0.5% SDS at room temperature. Hybridizing plaques were isolated and a 4.8kb SacI DNA fragment was isolated from one clone that hybridized to the oligonucleotide probe mixture.

A segment of the 4.8 kb SacI DNA fragment was sequenced, Sanger et al., Proc. Natl. Acad. Sci. U.S.A. 74:5463 (1977), [Fig. 1] and found to contain an open reading frame of 1236 base pairs encoding a protein of approximately 45,400 molecular weight. Within this open reading frame was a section that corresponded exactly to the amino acid sequence determined from the cytochrome P450soy tryptic peptide described above. The NH_2-terminal sequence of the open reading frame starting with amino acid 4 is the same as the amino acid sequence determined for P450soyΔ (other than a serine to cysteine change at amino acid 30 of the open reading frame). We have named the gene encoding the P450soy protein soyC. Five nucleotides downstream of the stop codon for soyC, another open reading frame of 65 amino acids was identified. This open reading frame shows 40-50% identity to the previously identified ferredoxins of *Streptomyces griseolus*, Ferredoxin-1 and Ferredoxin-2, encoded by the suaB and subB genes respectively. O'Keefe et al., Biochemistry 30:447 (1991). The gene encoding this apparent ferredoxin-like protein from *Streptomyces griseus* is designated soyB and the protein, ferredoxin-soy.

COMPARATIVE EXAMPLE

Nonconstitutive Expression of SoyC and SoyB in *Streptomyces lividans* From the SoyP Promoter

The 4.8kb SacI DNA fragment containing the soyC and the soyB genes was cloned (Maniatis et al. 1982) into

SacI cleaved pBluescript ks vector (Stratagene Inc., San Diego, CA) generating plasmid pMM001 (Figure 3) and into SacI cleaved pUC19, Yanisch-Peron et al., Gene 33:103(1985), generating plasmid pMM005 (Figure 5). The 4.8 kb SacI insert was then removed from pMM001 and cloned into the SacI site of pCAO200, Omer et al., J. Bacteriol. 170:2174 (1988) generating pMM002 (Figure 5). The plasmid pMM002 was transformed into *Streptomyces lividans* generating *Streptomyces lividans* MM002 (Figure 5).

Two independent strains of *Streptomyces lividans MM002* and one of *Streptomyces lividans* C200 containing the vector pCAO200 were each grown in 2 x 25 ml YEME medium containing 8.5% sucrose for approximately 60 hrs at 30°C . One of each of these cultures was subcultured in 100ml of YEME medium containing 4.25% sucrose and the other in 100ml of 5 x SBG medium. After growth at 30°C for 48 hrs, an additional 100 ml of growth medium was added and the cells grown for an additional 3 hrs. The cells were harvested and processed as described in the Material and Methods to obtain soluble protein extracts of each of the strains grown in the two different media. A ten microgram sample of each protein was analyzed for the presence of cytochrome P450soy by Western blot analysis.

In Figure 4, high levels of P450soy are seen only in the lanes containing purified P450soy protein and in *Streptomyces lividans* MM002 that has been grown in 5 x SBG. Much lower levels are seen when *Streptomyces lividans* MM002 was grown in YEME. Thus in *Streptomyces lividans* expression of P450soy from the 4.8kb SacI *Streptomyces griseus* (ATCC 13272) DNA fragment was induced by soybean flour as it is in *Streptomyces griseus*. This is different from the cytochrome P450 taken from *Streptomyces griseolus*. The genes for the

two sulfonylurea inducible cytochromes P450 in *Streptomyces griseolus* when transformed into *Streptomyces lividans* are constitutively expressed and do not require the presence of inducers.

EXAMPLE 1

Recombinant *Streptomyces Lividans* that constitutively express cytochrome P450

In order to constitutively express cytochrome P450soy in *Streptomyces lividans*, the transcriptional promoter, suaP, from the suaC and suaB genes of *Streptomyces griseolus* was cloned upstream of the soyC and soyB genes. suaP is located upstream of the *Streptomyces griseolus* (ATCC 11796) suaC gene and is located on a 0.6kb EcoRI-BamHI fragment of pCAO302. Omer et al., J. Bacteriol. 172:3335 (1990). An EcoRI site was introduced 23bp upstream of the ATG start codon of soyC of *Streptomyces griseus* by performing a polymerase chain reaction. Mullis, K. et al., Cold Spring Harbor Symp. Quant. Biol. 51:263 (1986). A pair of primers were used to carry out PCR on the soyC gene. One oligonucleotide 5'CAGAATTCGCACTGCGAGGCGAC 3' (Sequence ID NO. 8) contained 15 base pairs upstream of the soyC gene along with an EcoRI site near its 5' end. The other oligonucleotide was 5' GATCAGCGCGCCCAGGTACTCC 3' (SEQUENCE ID NO. 9) and is homologous to a region adjacent to an XhoI site within the soyC gene. When these two oligonucleotides were used to amplify soyC using pMM001 as template an approximately 0.67kb fragment was amplified. The conditions used for amplification of this DNA were as follows: 10mM Tris-Cl pH 8.3, 0.05M KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each dATP, dTTP, dCTP and 0.05mM dGTP plus 0.15 mM 7-deaza-dGTP. Each oligonucleotide was used at 1mM, 10ng pMM001 template and 2.5 units of Taq polymerase was used

in a 100 µl reaction. The temperatures used for amplification were:

1. 100°C, 2 min; 92°C, 5 min (add Taq polymerase); 72°C, 2 min; 1 cycle;
2. 96°C, 1 min; 47°C, 1 min; 72°C, 2 min; 5 cycles;
3. 96°C, 1 min; 65°C, 1 min; 72°C, 2 min; 25 cycles; and
4. 72°C, 5 min; 1 cycle.

The amplified DNA was precipitated with 2M ammonium acetate plus 1 volume isopropanol overnight at −20°C . The precipitate was pelleted at 12,000 x g at 4°C for 15 min, washed twice with 70% ethanol and resuspended in H_2O.

The generation of pMM004 occurred as follows. The amplified 0.67kb fragment of DNA was cloned into the EcoRI site of PUC19, Yanisch-Peron et al., Gene 33:103 (1985), after adding EcoRI linkers (New England Biolabs, Beverly, MA) to the amplified DNA (Maniatis et al. 1982) generating plasmid pMM003. The 4.8kb SacI fragment containing soyC and soyB was removed from pMM001 and inserted into pUC19 at the SacI site generating plasmid pMM005. A three way ligation was performed between 1) a 0.67kb EcoRI-XhoI fragment of pMM003 containing one end of soyC with the added EcoRI site, 2) a 0.6 kb EcoRI-BamHI fragment of pCA0302 containing suaP, and 3) an approximately 6.0kb BamHI-XhoI fragment of pMM005 containing part of the soyC gene, the soyB gene and pUC19. The resulting vector is pMM004(see Fig. 5).

The generation of plasmid pMM007 occurred as follows. The plasmid pIJ702-322 was made in *E. coli* by ligating SphI cut pIJ702, Katz et al., J. Gen. Microbiol. 129:2703 (1983), to SphI cut pBR322. Hoffman, K. H., et al., J. Basic Microbiol. 30:37 (1990). pIJ702 can replicate in *Streptomyces lividans*,

while pBR322 replicates in *E. coli*. pIJ702-322 was cut with SacI and ligated to a 4.1kb SacI DNA fragment of pMM004 that contains suaP linked to soyC, soyB to generate pMM006. pMM006 was cut with SphI, and self-ligated under dilute conditions (~3 µg/ml) (Maniatis et al. 1982) to separate the pBR322 part of the plasmid from the rest of pMM006 and generating plasmid pMM007 which is capable of replicating in *Streptomyces lividans* but not *E. coli*. This ligated DNA was used to transform *Streptomyces lividans* generating *Streptomyces lividans* MM007 (see Figure 6).

Transformation of *Streptomyces lividans* was performed as described by Hopwood, D. A. et al., Genetic Manipulation of Streptomyces: A Laboratory Manual, The John Innes Foundation, Norwich, UK (1985). Cloning of DNAs in *E. coli* was performed as described by Maniatis, T. et al., A Guide to Molecular Cloning, Cold Spring Harbor, (1982). Restriction enzymes and DNA modification enzymes were obtained from New England Bioloabs Inc. Beverly, MA. Taq DNA polymerase were obtained from Cetus-Perkin Elmer Inc.

25 ml cultures of *Streptomyces lividans* transformed with pIJ702 and *Streptomyces lividans* MM007 were grown at 30°C for 60 hrs. in YEME medium or 5x SBG medium with 5 µg/ml of thiostrepton. *Streptomyces griseus* was grown at 30°C for 60 hrs. in YEME medium or 5 x SBG medium. After 60 hrs., 10 ml of fresh medium was added to each culture and the cultures were incubated for an additional 2 hrs. 45 min. with shaking at 30°C. The cultures were harvested and soluble protein fractions were isolated from each culture. A Western blot of proteins from the cultures was performed to detect expression of cytochrome P450soy. As can be seen in Figure 7, expression of cytochrome P450soy in *Streptomyces lividans* MM007 is at least as high as in

Streptomyces griseus and addition of soybean flour is not required for high level expression of P450soy in *Streptomyces lividans* MM007.

EXAMPLE 2

Streptomyces lividans MM007 was grown (25 ml culture) according to the two-stage fermentation protocol. The medium used for cultivation of the organism was YEME containing: yeast extract (3 g/l); peptone (5 g/l); malt extract (3 g/l); glucose (10 g/l); sucrose (340 g/l); $MgCl_2$ from a 2.5 M solution (2 ml/L). Thiostrepton was added to insure the maintenance of the plasmid in the organism (62.5 microliter from a stock solution of 4 mg/ml). The first stage cultures were started from spore suspensions of *Streptomyces lividans* MM007. After 3 days of growth on stage one, a 20% inoculum was used to start a stage two culture in fresh YEME medium. After 24 hours, 3 ml of HMPA was added to the culture and at 24 hr and 48 hr 5 ml samples were drawn and extracted with 3 ml of ethyl acetate. The mixture was vigorously extracted by vortexing and allowing the organic and aqueous layers to separate. The organic layer was transferred to a glass vial and evaporated under a stream of nitrogen.

Gas chromatography and mass spectrophotometric (GC/MS) analysis (using a Carbowax capillary column (J. W. Scientific, Folsum, CA), 20 m, with a temperature gradient of 60 to 200 at 10° per min) indicate degradation of HMPA by *Streptomyces lividans* MM007. The presence of pentamethyl-phosphoramide (PMPA) and other metabolites were identified. Gas chromatographic analysis was performed on a Varian Vista 6000, Varian Co., Palo Alto, CA. Mass spectrophotometric analysis was performed on a VG 7070 HS Micromass Mass Spectrometer, Micromass Ltd., Manchester, U.K.

EXAMPLE 3

In another embodiment *Streptomyces lividans* MM007 was used as above with the exception that three, stage one cultures were centrifuged and the resultant cell paste was added to a single 25 ml culture flask containing fresh YEME medium. 0.2 ml of HMPA was immediately added to the second stage culture. Samples were taken as described above. GC/MS analysis demonstrated the presence of many different metabolites. The generation of PMPA and other metabolites by *Streptomyces lividans* MM007 when exposed to HMPA is a strong indication of the ability of *Streptomyces lividans* MM007 to degrade HMPA.

The metabolism of HMPA in Example 3 indicates the utility of *Streptomyces lividans* MM007 for bioremediation of several compounds.

A control experiment was performed in which HMPA was not added to the cultures of *S. lividans* MM007. This culture was extracted as above and analyzed by GC/MS. Apart from one peak seen to be present in all control test samples, none of the peaks observed in HMPA samples and *S. lividans* MM007 were present in control. This data confirms that the peaks observed in test samples were derived from metabolism of HMPA by *S. lividans* MM007.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention and, without departing from the spirit and scope thereof, can make various changes and modifications to the invention to adapt it to various usages and conditions. *Streptomyces lividans* MM007 is deposited with the American Type Culture Collection under the Budapest Treaty and has been given ATCC designation 68883. This strain will be maintained for a a period of at least thirty years after the date of

deposit, and for at least five years after the most recent request for a sample.

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 21, line 34 of the description [1]

A. IDENTIFICATION OF DEPOSIT [3]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit [5]	Accession Number [6]
13 December 1991 (13.12.91)	ATCC 68883

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SARIASLANI, SIMA

(ii) TITLE OF INVENTION: CONSTITUTIVE EXPRESSION OF P450SOY AND FERREDOXIN-SOY IN STREPTOMYCES

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: E. I. DU PONT DE NEMOURS AND COMPANY
(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: USA
(F) ZIP: 19898

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.50 inch, 1.0 MB
(B) COMPUTER: Macintosh
(C) OPERATING SYSTEM: Macintosh System, 6.0
(D) SOFTWARE: Microsoft Word, 4.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: GALLEGOS, R. THOMAS
(B) REGISTRATION NUMBER: 32,692
(C) REFERENCE/DOCKET NUMBER: CR-9000-A

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 302-892-7342
(B) TELEFAX: 302-892-7949

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Thr Thr Asp Pro Ala Arg Gln Asn Leu Asp Pro Thr Ser Pro Ala Pro
1 5 10 15

Ala Thr Ser Phe Pro Gln Asp Arg Gly Ser Pro Tyr His Pro
20 25 30

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

His His Leu Ala Phe Gly Phe Gly Val His Gln Cys Leu Gly Gln Asn
1 5 10 15

Leu Ala Arg

(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTCGGGGTGC ACCAGTGCCT 20

(2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTCGGGGTCC ACCAGTGCCT 20

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTCGGCGTGC ACCAGTGCCT 20

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTCGGCGTCC ACCAGTGCCT 20

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Phe Gly Val His Gln Cys Leu
1 5

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAGAATTCGC ACTGCGAGGC GAC 23

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: ingle
(D) TOPOLOGY: lir ar
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GATCAGCGCG CCCAGGTACT CC 22

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1735 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATATCTTTAC TACGAACAAC ACCCCTTGGT GGGCATACGA ACAACACCGG CCAGATCCAC 60

GGGCCCGCCG AGCTGGCCGG TCTACCCGTC GACCAGATAG GTGCCTGAGG CATCTAATAG 120

TGAAGAAGCG CGGAACGACC GGCTCCGCGC GCACGACCGA GCACTGCGAG GCGACCCGAT 180

CCCATGACGG AATCCACGAC GGACCCGGCC CGCCAGAACC TCGACCCCAC CTCCCCGGCC 240

CCCGCGACGT CCTTCCCGCA GGACCGCGGG TGCCCCTACC ACCCGCCCGC CGGGTACGCA 300

CCGCTGCGCG AGGGCCGCCC GCTGAGCCGG GTCACCCTCT TCGACGGACG CCCGGTCTGG 360

GCGGTCACCG GGCACGCCCT GGCCCGTCGG CTACTGGCGG ACCCGCGGCT CTCCACCGAC 420

CGCAGCCACC CGGACTTCCC CGTCCCGGCC GAGCGGTTCG CCGGCGCGCA GCGGCGCCGC 480

GTCGCTCTGC TCGGCGTCGA CGACCCCGAG CACAACACCC AGCGCAGGAT GCTCATCCCG 540

ACCTTCTCGG TGAAGCGGAT CGGCGCGCTC CGCCCGCGTA TCCAGGAGAC CGTGGACCGG 600

CTCCTCGACG CGATGGAGCG ACAAGGGCCC CCGGCCGAAC TGGTGAGCGC GTTCGCCCTG 660

CCGGTGCCGT CGATGGTGAT CTGTGCTCTG CTCGGCGTGC CCTACGCCGA CCACGCGTTC 720

TTCGAGGAAC GCTCGCAGCG ACTCCTGCGC GGCCCGGGAG CCGACGATGT GAACAGGGCC 780

CGCGACGAAC TCGAGGAGTA CCTGGGCGCG CTGATCGACC GCAAGAGGGC GGAGCCGGGT 840

GACGGCCTCC TGGACGAGCT GATCCACCGG GACCACCCGG ACGGACCGGT CGACCGCGAA 900

CAGCTGGTCG CCTTCGCCGT CATCCTGCTC ATCGCCGGGC ACGAGACGAC GGCGAACATG 960

ATCTCGCTCG GCACGTTCAC GCTGCTGAGC CACCCCGAAC AGCTGGCGGC GCTGCGGGCC 1020

GGCGGGACGA GCACCGCCGT GGTGGTCGAG GAGCTGCTGC GGTTCCTCTC CATCGCCGAG 1080

GGCCTCCAGC GCCTGGCGAC CGAGGACATG GAGGTCGACG GGGCGACGAT CCGCAAGGGG 1140

GAGGGCGTGG TCTTCTCGAC CTCGCTGATC AACCGCGACG CCGACGTGTT CCCCCGGGCC 1200

GAGACACTCG ACTGGGACCG CCCCGCCCGC CATCACCTCG CCTTCGGCTT CGGAGTCCAC 1260

CAGTGCCTGG GCCAGAACCT GGCCCGCGCC GAGCTGGACA TCGCGATGCG CACCCTGTTC 1320

GAGCGGCTTC CCGGGCTCAG GCTCGCCGTA CCCGCGCACG AGATCCGTCA CAAGCCGGGG 1380

GACACGATCC AGGGCCTCCT CGACCTGCCC GTGGCCTGGT GAGCGGCGTG GGAGTCCAGG 1440

TCGACAAGGA ACGCTGTGTG GGCGCCGGCA TGTGTGCGCT GACCGCGCCG GACGTCTTCA 1500

CCCAGGACGA CGACGGTCTC AGCGAGGTGC TCCCCGGCCG GGAGGCGACG TCCGGGACCC 1560

ATCCGCTGGT GGGGGAGGCG GTACGGGCCT GCCCGGTGGG GGCGGTGGTC CTCTCCTCCG 1620

ACTGACGTCC CCCGGCACGG GGTTCGCCTC TTGCTGCCAT GGCTCGGCGC CGAGGTCAAC 1680

GACAGCAATC CCAGGGCATT TATGATGTCT TGATGCGATC TGTCCCTTGG TGGGC 1735

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 412 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Thr Glu Ser Thr Thr Asp Pro Ala Arg Gln Asn Leu Asp Pro Thr
1 5 10 15

Ser Pro Ala Pro Ala Thr Ser Phe Pro Gln Asp Arg Gly Cys Pro Tyr
20 25 30

His Pro Pro Ala Gly Tyr Ala Pro Leu Arg Glu Gly Arg Pro Leu Ser
35 40 45

Arg Val Thr Leu Phe Asp Gly Arg Pro Val Trp Ala Val Thr Gly His
50 55 60

Ala Leu Ala Arg Arg Leu Leu Ala Asp Pro Arg Leu Ser Thr Asp Arg
65 70 75 80

Ser His Pro Asp Phe Pro Val Pro Ala Glu Arg Phe Ala Gly Ala Gln
85 90 95

Arg Arg Arg Val Ala Leu Leu Gly Val Asp Asp Pro Glu His Asn Thr
100 105 110

Gln Arg Arg Met Leu Ile Pro Thr Phe Ser Val Lys Arg Ile Gly Ala
115 120 125

Leu Arg Pro Arg Ile Gln Glu Thr Val Asp Arg Leu Leu Asp Ala Met
130 135 140

```
Glu Arg Gln Gly Pro Pro Ala Glu Leu Val Ser Ala Phe Ala Leu Pro
145                 150                 155                 160

Val Pro Ser Met Val Ile Cys Ala Leu Leu Gly Val Pro Tyr Ala Asp
                165                 170                 175

His Ala Phe Phe Glu Glu Arg Ser Gln Arg Leu Leu Arg Gly Pro Gly
            180                 185                 190

Ala Asp Asp Val Asn Arg Ala Arg Asp Glu Leu Glu Glu Tyr Leu Gly
        195                 200                 205

Ala Leu Ile Asp Arg Lys Arg Ala Glu Pro Gly Asp Gly Leu Leu Asp
    210                 215                 220

Glu Leu Ile His Arg Asp His Pro Asp Gly Pro Val Asp Arg Glu Gln
225                 230                 235                 240

Leu Val Ala Phe Ala Val Ile Leu Leu Ile Ala Gly His Glu Thr Thr
                245                 250                 255

Ala Asn Met Ile Ser Leu Gly Thr Phe Thr Leu Leu Ser His Pro Glu
            260                 265                 270

Gln Leu Ala Ala Leu Arg Ala Gly Gly Thr Ser Thr Ala Val Val Val
        275                 280                 285

Glu Glu Leu Leu Arg Phe Leu Ser Ile Ala Glu Gly Leu Gln Arg Leu
    290                 295                 300

Ala Thr Glu Asp Met Glu Val Asp Gly Ala Thr Ile Arg Lys Gly Glu
305                 310                 315                 320

Gly Val Val Phe Ser Thr Ser Leu Ile Asn Arg Asp Ala Asp Val Phe
                325                 330                 335

Pro Arg Ala Glu Thr Leu Asp Trp Asp Arg Pro Ala Arg His His Leu
            340                 345                 350

Ala Phe Gly Phe Gly Val His Gln Cys Leu Gly Gln Asn Leu Ala Arg
        355                 360                 365

Ala Glu Leu Asp Ile Ala Met Arg Thr Leu Phe Glu Arg Leu Pro Gly
    370                 375                 380

Leu Arg Leu Ala Val Pro Ala His Glu Ile Arg His Lys Pro Gly Asp
385                 390                 395                 400

Thr Ile Gln Gly Leu Leu Asp Leu Pro Val Ala Trp
                405                 410
```

WHAT IS CLAIMED IS:

1. A purified nucleic acid fragment comprising a *Sac*I restriction fragment which comprises the cytochrome P450soy gene from *Streptomyces griseus*.

2. A purified nucleic acid fragment according to Claim 1 further comprising a promoter region operably linked to the 5' end of the *Sac*I fragment.

3. A purified nucleic acid fragment according to Claim 2 wherein the promoter region is from *Streptomyces griseolus*, *Streptomyces coelicolor*, *Streptomyces azureus* or *Streptomyces lividans*.

4. A purified nucleic acid fragment according to Claim 1 further comprising a region coding for ferredoxin soy.

5. A nucleic acid fragment comprising:

a) a first region coding for a promoter cloned from *Streptomyces griseolus* which is capable of constitutively transcribing soyC and soyB in *Streptomyces* bacteria;

b) a second region coding for *Streptomyces griseus* cytochrome P450 soy, said second region being upstream of the ferredoxin-soy coding region; and

c) a third region coding for Streptomyces griseus ferredoxin soy, said third region operably linked to and downstream of the promoter region.

6. A recombinant vector expressible in *Streptomyces* comprising the nucleic acid fragment of Claim 5.

7. A recombinant *Streptomyces* comprising the vector of Claim 6.

8. *Streptomyces lividans* containing the vector pMM007 described in Figure 6.

9. A method of constitutive production of cytochrome P450 soy, comprising:

a) growing the *Streptomyces* of Claim 8 in appropriate medium; and

b) isolating cytochrome P450 soy.

10. A method of biochemical oxidation, comprising:

a) growing a *Streptomyces* organism containing pMM007 in the presence of a chemical to be metabolized; and

b) obtaining one or more products resulting from the metabolism of the chemical.

11. The method of Claim 10 wherein the chemical oxidized is taken from the group consisting of hexamethylphosphoramide (HMPA), pentamethylphosphoramide (PMPA), tetramethylphosphoramide (TetraMPA), trimethylphosphoramide (TriMPA), 7-ethoxycoumarin; precocene II; anisole; benzene; biphenyl; chlorobenzene; coumarin; naphthalene; trans-stilbene; toluene; glaucine; 10,11-dimethoxyaporphine; papaverine; d-tetrandrine; thalicarpine; bruceantin; vindoline; dihydrovindoline; leurosine; and codeine.

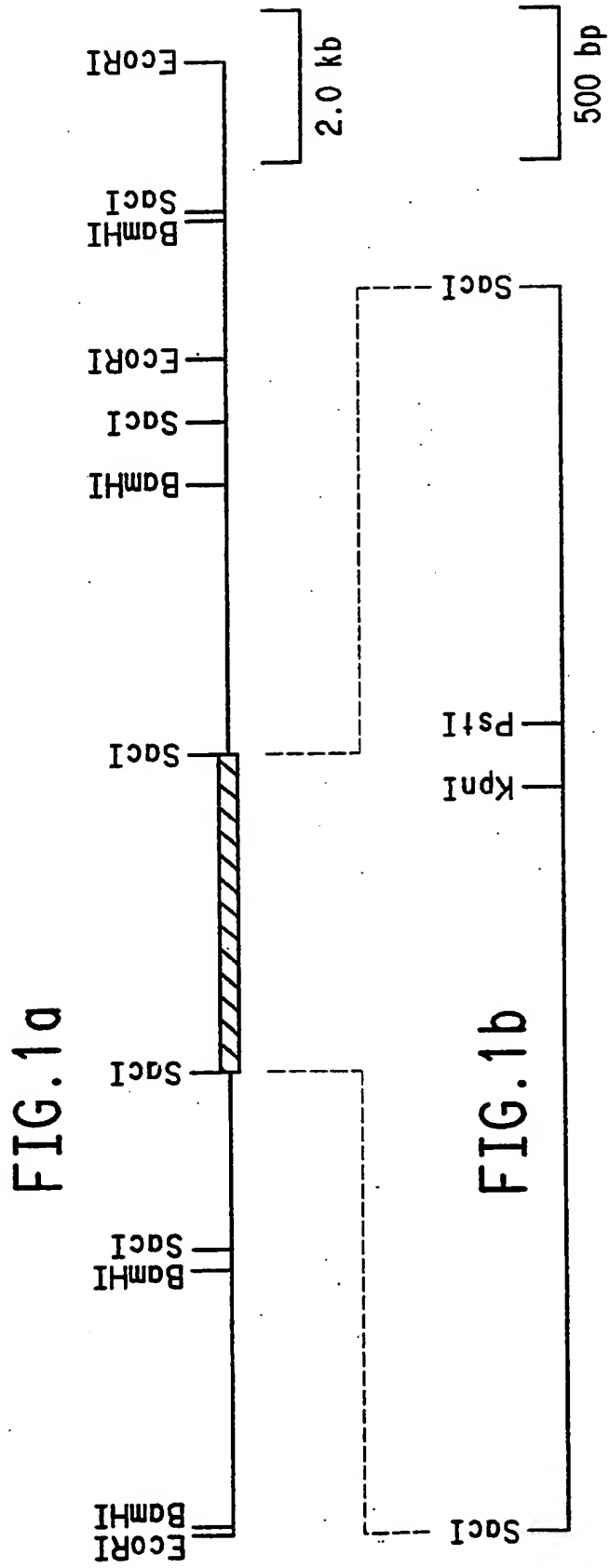

FIG. 1a

FIG. 1b

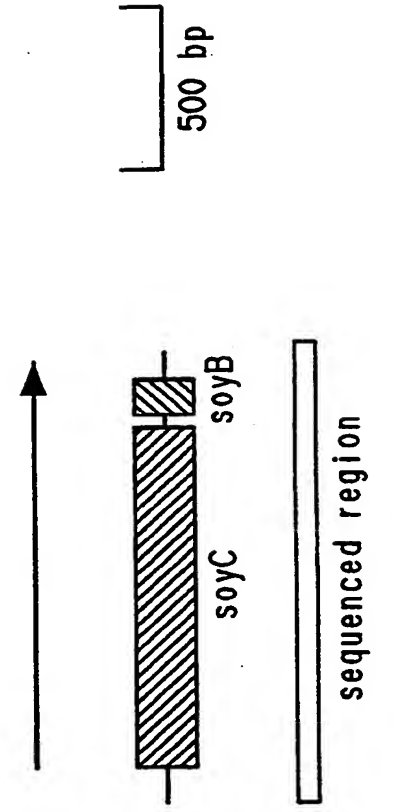

FIG. 1c

SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
TACGAACAAC ACCGGCCAGA TCCACGGGCC CGCCGAGCTG GCCGGTCTAC CCGTCGACCA   60

GATAGGTGCC TGAGGCATCT AATAGTGAAG AAGCGCGGAA CGACCGGCTC CGCGCGCACG  120

ACCGAGCACT GCGAGGCGAC CCGATCCCAT GACGGAATCC ACGACGGACC CGGCCCGCCA  180

GAACCTCGAC CCCACCTCCC CGGCCCCCGC GACGTCCTTC CCGCAGGACC GCGGGTGCCC  240

CTACCACCCG CCCGCCGGGT ACGCACCGCT GCGCGAGGGC CGCCCGCTGA GCCGGGTCAC  300

CCTCTTCGAC GGACGCCCGG TCTGGGCGGT CACCGGGCAC GCCCTGGCCC GTCGGCTACT  360

GGCGGACCCG CGGCTCTCCA CCGACCGCAG CCACCCGGAC TTCCCCGTCC CGGCCGAGCG  420

GTTCGCCGGC GCGCAGCGGC GCCGCGTCGC TCTGCTCGGC GTCGACGACC CCGAGCACAA  480

CACCCAGCGC AGGATGCTCA TCCCGACCTT CTCGGTGAAG CGGATCGGCG CGCTCCGCCC  540

GCGTATCCAG GAGACCGTGG ACCGGCTCCT CGACGCGATG GAGCGACAAG GGCCCCCGGC  600

CGAACTGGTG AGCGCGTTCG CCCTGCCGGT GCCGTCGATG GTGATCTGTG CTCTGCTCGG  660

CGTGCCCTAC GCCGACCACG CGTTCTTCGA GGAACGCTCG CAGCGACTCC TGCGCGGCCC  720

GGGAGCCGAC GATGTGAACA GGGCCCGCGA CGAACTCGAG GAGTACCTGG GCGCGCTGAT  780

CGACCGCAAG AGGGCGGAGC CGGGTGACGG CCTCCTGGAC GAGCTGATCC ACCGGGACCA  840

CCCGGACGGA CCGGTCGACC GCGAACAGCT GGTCGCCTTC GCCGTCATCC TGCTCATCGC  900
```

FIG. 2a

```
CGGGCACGAG ACGACGGCGA ACATGATCTC GCTCGGCACG TTCACGCTGC TGAGCCACCC  960

CGAACAGCTG GCGGCGCTGC GGGCCGGCGG GACGAGCACC GCCGTGGTGG TCGAGGAGCT 1020

GCTGCGGTTC CTCTCCATCG CCGAGGGCCT CCAGCGCCTG GCGACCGAGG ACATGGAGGT 1080

CGACGGGGCG ACGATCCGCA AGGGGGAGGG CGTGGTCTTC TCGACCTCGC TGATCAACCG 1140

CGACGCCGAC GTGTTCCCCC GGGCCGAGAC ACTCGACTGG GACCGCCCCG CCCGCCATCA 1200

CCTCGCCTTC GGCTTCGGAG TCCACCAGTG CCTGGGCCAG AACCTGGCCC GCGCCGAGCT 1260

GGACATCGCG ATGCGCACCC TGTTCGAGCG GCTTCCCGGG CTCAGGCTCG CCGTACCCGC 1320

GCACCAGATC CGTCACAAGC CGGGGGACAC GATCCAGGGC CTCCTCGACC TGCCCGTGGC 1380

CTGGTGAGCG GCGTGGGAGT CCAGGTCGAC AAGGAACGCT GTGTGGGCGC CGGCATGTGT 1440

GCGCTGACCG CGCCGGACGT CTTCACCCAG GACGACGACG GTCTCAGCGA GGTGCTCCCC 1500

GGCCGGGAGG CGACGTCCGG GACCCATCCG CTGGTGGGGG AGGCGGTACG GGCCTGCCCG 1560

GTGGGGGCGG TGGTCCTCTC CTCCGACTGA CGTCCCCCGG CACGGGGTTC GCCTCTTGCT 1620

GCCATGGCTC GGCGCCGAGG TCAACGACAG CAATCCCAGG GCATTTATGA TGTCTTGATG 1680

CGATCTGTCC CTTGGTGGGC                                            1700
```

FIG.2b

FIG.2c

SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Thr Glu Ser Thr Thr Asp Pro Ala Arg Gln Asn Leu Asp Pro Thr
1               5                  10                  15

Ser Pro Ala Pro Ala Thr Ser Phe Pro Gln Asp Arg Gly Cys Pro Tyr
            20                  25                  30

His Pro Pro Ala Gly Tyr Ala Pro Leu Arg Glu Gly Arg Pro Leu Ser
        35                  40                  45

Arg Val Thr Leu Phe Asp Gly Arg Pro Val Trp Ala Val Thr Gly His
    50                  55                  60

Ala Leu Ala Arg Arg Leu Leu Ala Asp Pro Arg Leu Ser Thr Asp Arg
65                  70                  75                  80

Ser His Pro Asp Phe Pro Val Pro Ala Glu Arg Phe Ala Gly Ala Gln
                85                  90                  95

Arg Arg Arg Val Ala Leu Leu Gly Val Asp Asp Pro Glu His Asn Thr
            100                 105                 110

Gln Arg Arg Met Leu Ile Pro Thr Phe Ser Val Lys Arg Ile Gly Ala
        115                 120                 125

Leu Arg Pro Arg Ile Gln Glu Thr Val Asp Arg Leu Leu Asp Ala Met
    130                 135                 140

Glu Arg Gln Gly Pro Pro Ala Glu Leu Val Ser Ala Phe Ala Leu Pro
145                 150                 155                 160
```

FIG.2d

```
Val Pro Ser Met Val Ile Cys Ala Leu Leu Gly Val Pro Tyr Ala Asp
                165                 170                 175

His Ala Phe Phe Glu Glu Arg Ser Gln Arg Leu Leu Arg Gly Pro Gly
            180                 185                 190

Ala Asp Asp Val Asn Arg Ala Arg Asp Glu Leu Glu Glu Tyr Leu Gly
        195                 200                 205

Ala Leu Ile Asp Arg Lys Arg Ala Glu Pro Gly Asp Gly Leu Leu Asp
    210                 215                 220

Glu Leu Ile His Arg Asp His Pro Asp Gly Pro Val Asp Arg Glu Gln
225                 230                 235                 240

Leu Val Ala Phe Ala Val Ile Leu Leu Ile Ala Gly His Glu Thr Thr
                245                 250                 255

Ala Asn Met Ile Ser Leu Gly Thr Phe Thr Leu Leu Ser His Pro Glu
            260                 265                 270

Gln Leu Ala Ala Leu Arg Ala Gly Gly Thr Ser Thr Ala Val Val Val
        275                 280                 285

Glu Glu Leu Leu Arg Phe Leu Ser Ile Ala Glu Gly Leu Gln Arg Leu
    290                 295                 300

Ala Thr Glu Asp Met Glu Val Asp Gly Ala Thr Ile Arg Lys Gly Glu
305                 310                 315                 320
```

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```
Gly Val Val Phe Ser Thr Ser Leu Ile Asn Arg Asp Ala Asp Val Phe
325                 330                 335

Pro Arg Ala Glu Thr Leu Asp Trp Asp Arg Pro Ala Arg His His Leu
340                 345                 350

Ala Phe Gly Phe Gly Val His Gln Cys Leu Gly Gln Asn Leu Ala Arg
355                 360                 365

Ala Glu Leu Asp Ile Ala Met Arg Thr Leu Phe Glu Arg Leu Pro Gly
370                 375                 380

Leu Arg Leu Ala Val Pro Ala His Glu Ile Arg His Lys Pro Gly Asp
385                 390                 395                 400

Thr Ile Gln Gly Leu Leu Asp Leu Pro Val Ala Trp
                405                 410
```

FIG.2e

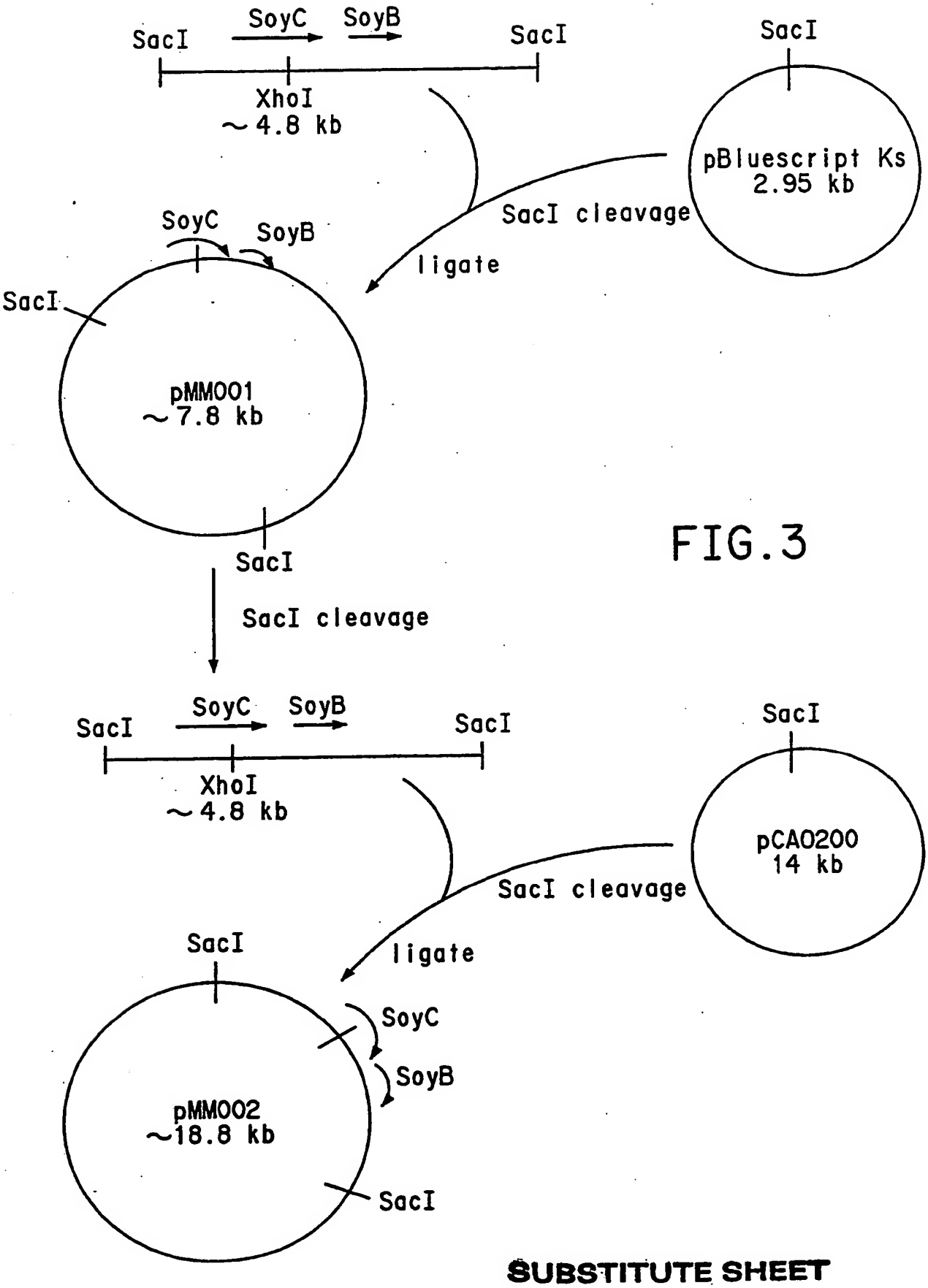

FIG.3

FIG.4

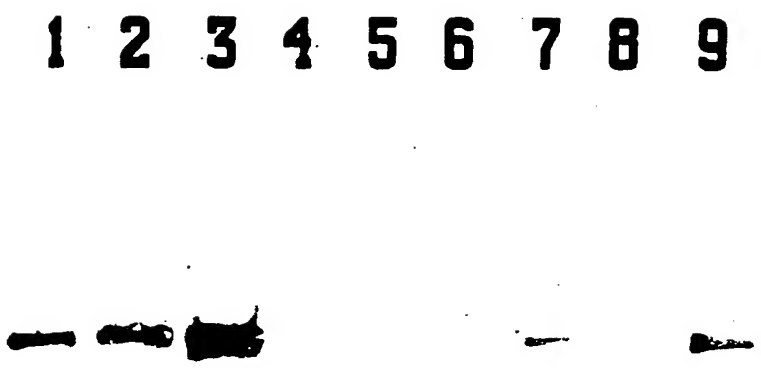

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FIG.5a

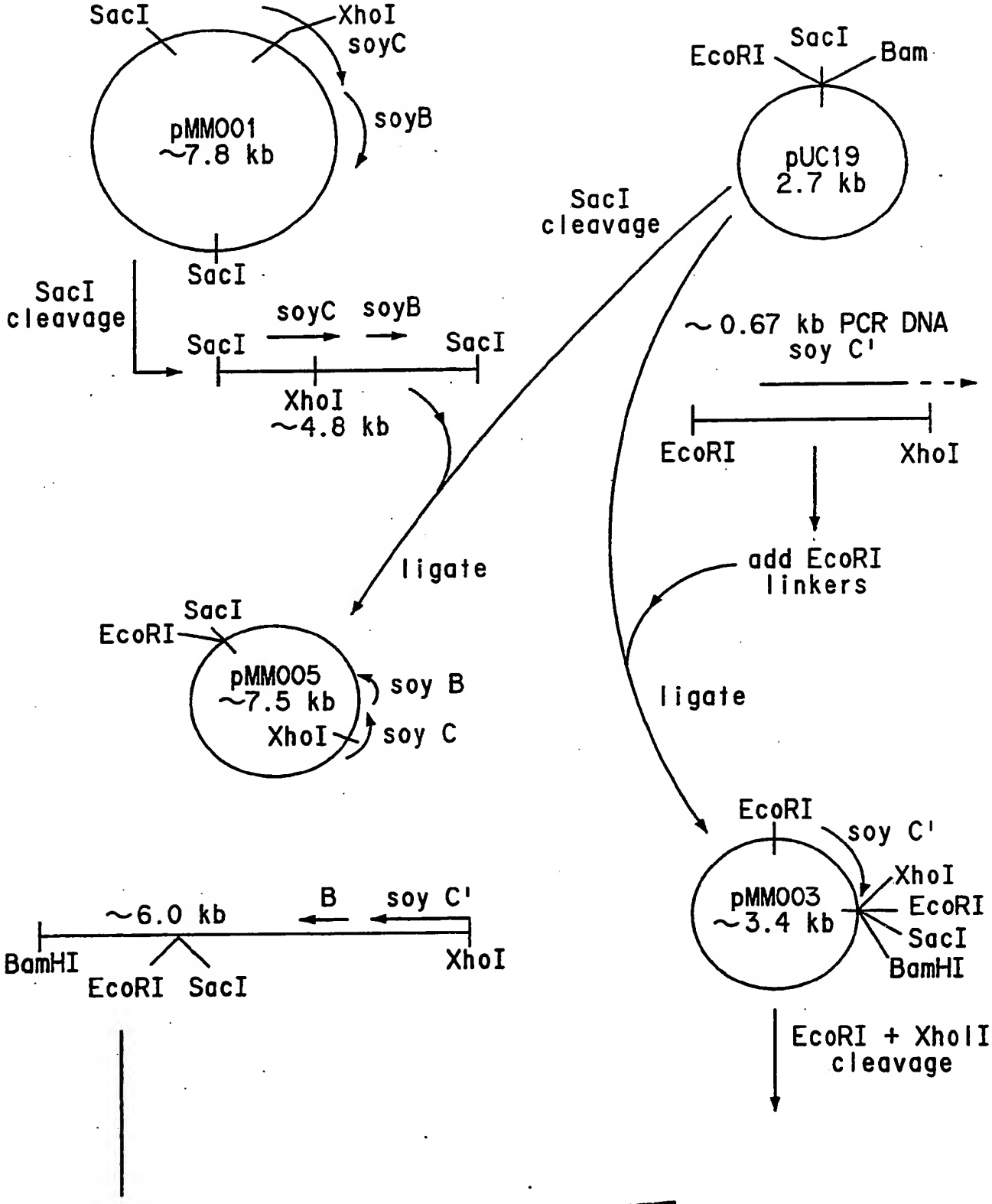

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FIG.5b

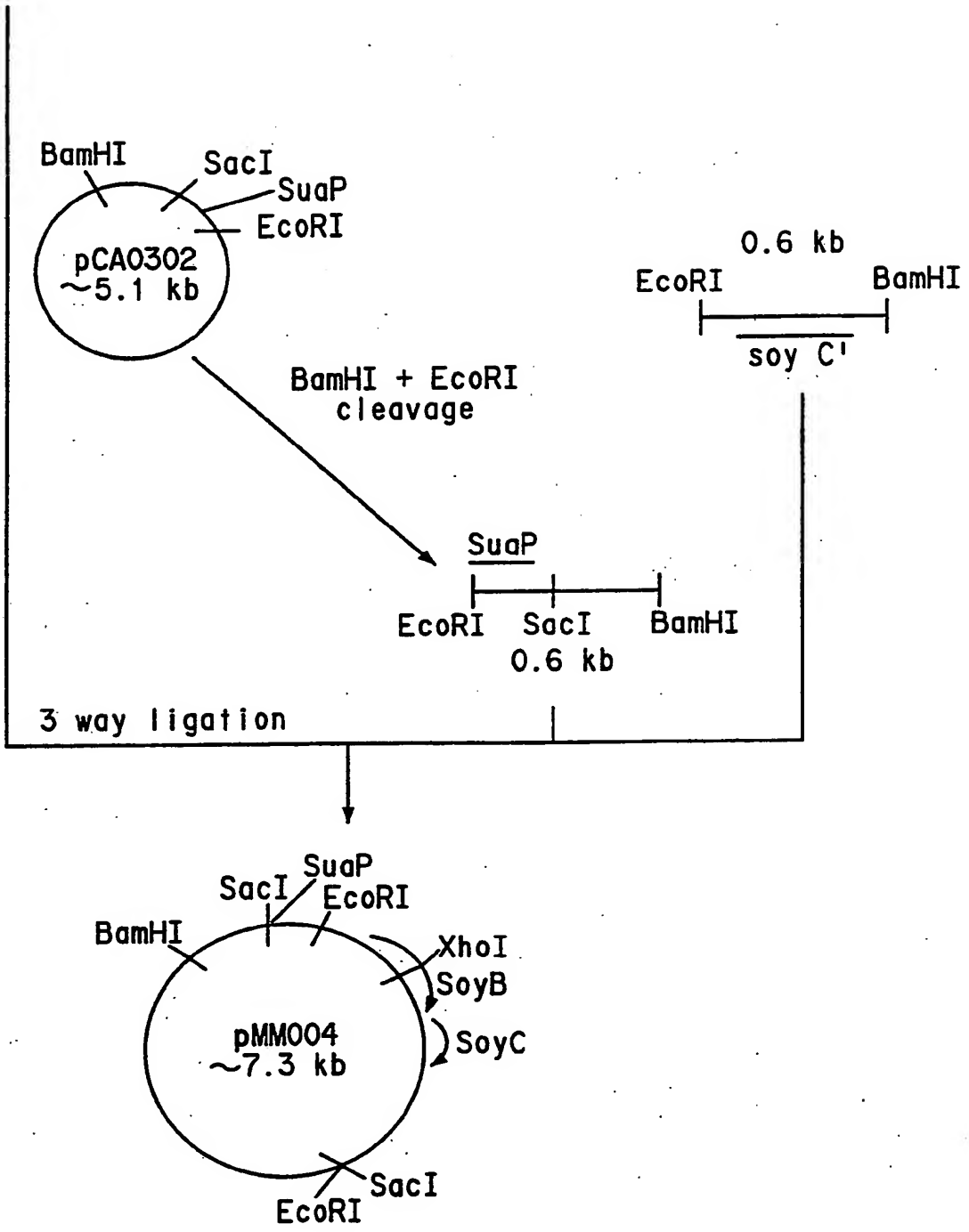

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FIG.6

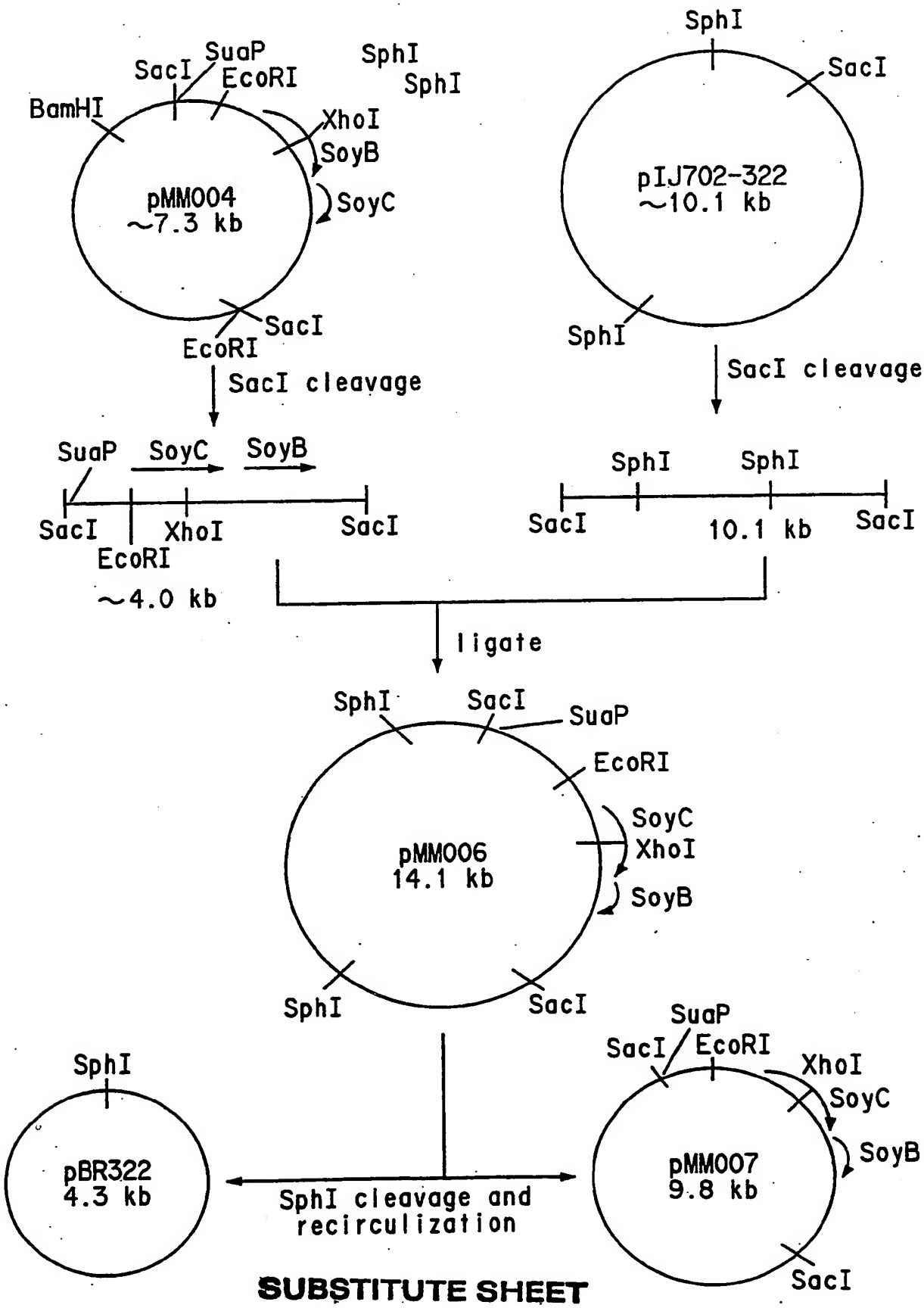

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FIG.7

1 2 3 4 5 6 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/10885

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/53; C12N15/76; C12P1/06; C12N1/21
//(C12N1/21,C12R1:465)C12P13/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	WO,A,9 103 561 (E. I. DU PONT DE NEMOURS) 21 March 1991 cited in the application see page 3, line 24 - line 28 see page 5, line 28 - page 6, line 27 see page 36, line 21 - page 40, line 13 ---	1-11
Y	JOURNAL OF BACTERIOLOGY vol. 171, no. 4, April 1989, pages 1781 - 1787 TROWER M. K. ET AL 'Purification and characterization of a soybean flour-induced cytochrome P-450 from Streptomyces griseus' cited in the application see the whole document ---	1-11

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 APRIL 1993	16. 04. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LE CORNEC N.D.R.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	DEVELOPMENT IN INDUSTRIAL MOCROBIOLOGY vol. 30, 1989, pages 161 - 171 SARIASLANI F. ET AL 'Xenobiotic tranformations by Streptomyces griseus' cited in the application see the whole document	10-11
Y	BIOCHEMICA ET BIOPHYSICA ACTA vol. 1037, no. 3, 1 March 1990, pages 290 - 296 M. K. TROWER ET AL 'Primary structure of a 7fe ferredoxin from Streptomyces griseus' see the whole document	1-11
P,X	MOLECULAR MICROBIOLOGY vol. 6, no. 15, August 1992, EDINBURGH;UK. pages 2125 - 2134 M. K. TROWER ET AL 'Cloning, nucleotide sequence determination and expression of the genes encoding cytochrome P-450soy(soyC) and ferredoxinsoy (soyB) from Streptomyces griseus' see the whole document	1-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9210885
SA 68252

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 07/04/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9103561	21-03-91	AU-A- 6272990	08-04-91
		CA-A- 2065439	12-03-91
		JP-T- 5500002	14-01-93